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Association of simple sequence repeat (SSR) markers with submergence tolerance in diverse populations of perennial ryegrass

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ABSTRACT

Submergence stress can cause the death of grass plants. Identification of the association between molecular markers and submergence tolerance-related traits facilitates an efficient selection of the tolerant cultivars for commercial production. A global collection of 99 diverse perennial ryegrass (*Lolium perenne* L.) accessions was evaluated for submergence tolerance and analyzed with 109 simple sequence repeat (SSR) markers. Submergence significantly reduced leaf color, chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR). Significant variations in these trait values were observed among the accessions under submerged conditions. Rapid linkage-disequilibrium (LD) decay was identified within 4 cM. The analysis of population structure (*Q*) identified four subpopulations in the collection, but no obvious relative kinship (*K*) was found. The *Q* model was the best to describe associations between SSR and traits, compared to the simple linear, *K*, and *Q*+*K* models. Fifteen SSR markers were associated with a reduction in leaf color, F_v/F_m, HT, and RGR under submergence stress using the *Q* model. These markers can be used for genetic improvement of submergence tolerance of perennial ryegrass after further validation. The diverse populations of perennial ryegrass is a valuable resource for association mapping of stress tolerance-related physiological traits.

1. Introduction

Agricultural production is largely influenced by climate variability and weather extremes. The increased frequency of flooding and other abiotic stresses negatively affects crop yields and livestock beyond the impacts of mean climate change [1]. Excess water in the soil reduces oxygen availability to the plant [2]. The extended deep submersion can cause death in plants due to a significant lack of oxygen required for energy production to sustain plant growth as well as due to an accumulation of toxic substances such as organic acids, NO_2^-, Mn^{2+}, Fe^{2+}, and H_2S [2,3]. Thus, development of more flood-tolerant cultivars is critical for enhancing sustainable production of crops including perennial grass species. A better understanding of plant physiological responses to flooding stress and identification of molecular markers associated with submergence tolerance will assist crop and grass breeders in developing flood-tolerant cultivars.

Growth and physiological responses of perennial grass species to flooding have not been well documented. Species, cultivars, water depth, and temperatures may all affect plant survival. Flooding stress decreases shoot and root dry weight and chlorophyll concentration [4–6], increases root cell membrane leakage [1], and alters antioxidant enzyme activities [7] of perennial grasses species. When roots of Kentucky bluegrass (*Poa pratensis* L.) were flooded with water for 30 d, the increased cell membrane leakage of roots ranged from 29% to 98% among ten cultivars, and the decreased root dry weight and root water-soluble carbohydrate concentration ranged from 27% to 60% and from 9% to 43%, respectively [4]. Water level at 15-, 5-, and 1-cm below the soil surface all significantly reduced leaf chlorophyll concentration and root dry weight of creeping bentgrass (*Agrostis palustris* L.) cultivars [6]. When plants were submerged, bahiagrass (*Paspalum notatum* Flügge) and bermudagrass (*Cynodon dactylon*) had higher shoot survival rate than did St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] and zoysiagrass (*Zoysia japonica*) following submersion, while centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.] did not survive under the same stress conditions [8].

Submergence stress either inhibits or enhances plant growth, depending on type of species and survival strategies [9]. In rice

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(*Oryza sativa* L.), complete submergence tolerance minimized shoot elongation and increased dry matter weight underwater [10]. However, stronger shoot elongation, longer leaf blade, and increased biomass and tiller production were also noted in flood tolerant perennial grass species from floodplains or lowland ecotypes, compared to meadow species or upland ecotypes with varied flooding responses under low-dynamic wetland to complete submergence conditions [11–13]. The variable growth and physiological responses of different perennial grass species and ecotypes to water excess stress provide an important basis for identifying molecular mechanisms of submergence tolerance and molecular markers linked to stress tolerance.

Molecular markers associated with a quantitative trait in plants are traditionally identified using a population derived from a biparental cross. The complementary method of association mapping, also known as linkage disequilibrium (LD) mapping, has been proved to be useful and powerful for genetic dissection of complex traits. Historically originating from human genetics [14], association mapping utilizes diverse plant populations in detecting the correlations between genes/markers and traits of interest [15]. An association mapping experiment generally consists of several steps: selecting population samples, determining the level and influence of population structure on the sample, phenotyping the population sample for traits of interest, genotyping the population either for candidate genes/regions or as a genome-wide scan, and testing the genotypes and phenotypes for their associations [16]. The pattern of LD decay can be used to determine whether a genome scan or candidate gene-association is more suitable for an association study [16]. Compared to linkage mapping in traditional biparental populations, association mapping results in a higher mapping resolution and evaluates a wide range of alleles rapidly [15]. Using this technique, candidate genes or molecular markers (e.g., simple repeat sequence) linked to important agronomic traits have been successfully identified in model plant and crop species. These traits include disease resistance of *Arabidopsis thaliana* [17], kernel size and milling quality of wheat (*Triticum aestivum* L.) [18], flowering time of perennial ryegrass [19] and *A. thaliana* [20], carotenoid content of maize (*Zea mays* L.) [21], iron deficiency chlorosis of soybean (*Glycine max* L.) [22], eating and cooking quality of rice [23], and salinity tolerance of barley (*Hordeum vulgare* L.) [24]. However, the use of association mapping in identifying links between genes or markers with complex traits such as abiotic stress tolerance is still in its infancy in plant species.

Perennial ryegrass is one of the most important cool-season grasses widely used in the turf and forage industry in North America, Asia, Europe, and Australia. Perennial ryegrass provides a good model for studying marker–trait association in perennial species due to its diploid genetics, existing resources of molecular markers, rapid stress responses, and wide distribution. The association results from perennial ryegrass may provide guideline for studying other popular perennial grass species that have more complex genomes. To date, the effects of submergence on the growth and physiology of perennial grasses, especially using the marker–trait association approach, have not been well documented. To the best of our knowledge, this study is the first report on LD mapping of submergence tolerance traits in perennial grass species. This research has revealed the phenotypic diversity of submergence tolerance and identified marker–trait association, which will benefit the development of resistant cultivars of perennial ryegrass and other important grass species.

2. Materials and methods

2.1. Plant materials and growing conditions

A total of 99 perennial ryegrass materials were used in this study. Among them, 93 accessions were obtained from the USDA National Plant Germplasm System at the Western Regional Plant Introduction Station in Pullman, WA, USA. These materials included 32 wild germplasm, 30 cultivars or cultivated accessions, and 31 accessions of uncertain status, according to germplasm bank classification (Table 1). Additional 6 turf-type commercial cultivars of perennial ryegrass were obtained from Turf-Seed Company, Gervais, OR and Scotts Inc., Marysville, OH, USA. All the accessions were confirmed as diploid by flow cytometry [25]. In 2008, a single seed from each accession was sown in a plastic pot (4-cm diameter, 9-cm deep) containing a sandy-loam soil with a pH of 6.9 in a greenhouse. Since then, each accession has been propagated through tillers multiple times. The grass propagations for this study were done on October 6, 2009 for Experiment 1 (Exp 1) and on December 23, 2009 for Experiment 2 (Exp 2). The duration of Exp 1 was from October 6 to November 21, 2009 and of Exp 2, from December 23, 2009 to February 5, 2010. After propagation, the plants were grown for 38 d prior to submergence stress. The plants were watered daily and fertilized once a week with a soluble fertilizer ($N–P_2O_5–K_2O$, 24–8–16) (Scotts Inc., Marysville, OH, USA) to provide 240 kg N ha^{-1}, 33 kg P ha^{-1}, 132 kg K ha^{-1} and micronutrients. During the growing periods, the average day air temperature was 19.3 ± 1.5 °C and the light intensity was about 300 μmol m^{-2} s^{-1} in the greenhouse with 8 h light period. Prior to submergence treatment, all the plants were cut to from 5 to 7 cm above the soil surface, then the plant height of each pot was recorded.

2.2. Submergence treatment

Submergence stress was imposed by submerging the grass pots in plastic containers (86 cm length × 38 cm width × 30 cm height) with tap water (pH 6.6). The water level was kept at 10 cm above the grass canopy at the beginning of the treatment. The control pots were placed in the same size containers without water. The stress treatments began on November 14 of 2009 for Exp 1 and January 29 of 2010 for Exp 2 and lasted 7 d for both experiments. Nutrients were not supplied to the plants and water was not changed during the treatment, but algae were removed manually if accumulated. During the periods of submergence stress, the air and water temperatures were 19.8 ± 1.0 °C and 17.5 ± 0.8 °C for Exp 1 and 19.9 ± 1.0 °C and 18.1 ± 1.4 °C for Exp 2, respectively.

2.3. Physiological parameters

The leaf color was rated visually on a scale of 1 (yellow) to 9 (dark green) for both the control and submerged plants. Leaf photochemical efficiency was determined by measuring the chlorophyll fluorescence (F_v/F_m) at dark on randomly selected leaves in each pot using a fluorescent meter (OS-30P, OPTI-Sciences, Hudson, NH, USA). At the end of the 7 d treatments, plant height was measured by recording the longest leaf blade. The maximum plant height (HT) increase during the treatment was obtained by subtracting the plant height prior to stress for each pot. The leaves corresponding to this HT were cut and the tissues were dried in an oven at 80 °C for 3 d. The relative growth rate (RGR) was calculated as dry weight per growing day (total 7 d) for both the control and the submerged plants. The percentage of reduction of all traits was used to indicate the plant submergence tolerance, calculated as [(control − submergence)/control] × 100.

2.4. DNA isolation and SSR analysis

Genomic DNA was extracted from the young leaves of each accession using a cetyltrimethyl ammonium bromide (CTAB) method [26]. DNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE, USA) and diluted to 15 ng μL^{-1} for PCR. A total of

Table 1
Origin and grouping information of perennial ryegrass accessions used in this study.

ID[a]	Accession	Origin	Status[b]	Q[c]	ID	Accession	Origin	Status	Q
1	187222[d]	Belgium	Uncertain	4	55	231604	Portugal	Uncertain	4
2	197270[d]	Finland	Cultivated	4	57	231619	Iran	Uncertain	2
4	202451[d]	Argentina	Wild	3	59	234779	Germany	Uncertain	3
5	204879[d]	Turkey	Wild	2	65	251224	Yugoslavia	Wild	3
7	206376[d]	Cyprus	Uncertain	1	68	265336	Sweden	Cultivar	3
9	229702[d]	Iran	Wild	2	72	265349	Ireland	Uncertain	3
10	231569[d]	Libya	Uncertain	4	77	274637	Poland	Uncertain	3
11	231578[d]	Algeria	Uncertain	1	78	Inspire	USA	Cultivar	3
12	231588[d]	Algeria	Uncertain	1	79	277846	Yugoslavia	Uncertain	2
13	231595[d]	Morocco	Uncertain	1	82	284823	Australia	Uncertain	3
14	231597	Greece	Uncertain	3	83	284826	Australia	Uncertain	3
15	231605[d]	Portugal	Uncertain	4	84	285101	Australia	Cultivar	3
16	251141[d]	Yugoslavia	Wild	2	85	287849	Spain	Uncertain	1
17	265344[d]	Ireland	Cultivar	3	89	303012	UK	Cultivar	3
18	265351[d]	Chile	Uncertain	3	92	303026	France	Cultivar	3
19	267059[d]	Poland	Uncertain	3	93	303027	Denmark	Cultivar	3
20	275660[d]	Australia	Cultivated	3	95	636643	Japan	Cultivated	3
21	287855[d]	Spain	Uncertain	1	96	303037	Sweden	Cultivar	3
22	298091[d]	Hungary	Wild	2	99	306292	Bolivia	Uncertain	4
23	303011[d]	UK	Cultivar	3	101	317452	Afghanistan	Wild	2
24	303022[d]	Netherlands	Cultivated	3	103	321397	Czech Republic	Uncertain	3
25	303031[d]	Netherlands	Cultivated	3	105	340104	Turkey	Uncertain	4
27	403889[d]	Canada	Cultivar	3	107	371952	Bulgaria	Uncertain	3
28	632542	Hungary	Cultivar	3	108	376878	New Zealand	Cultivar	3
29	418707[d]	Romania	Wild	3	109	384478	Poland	Cultivar	3
30	418714[d]	Italy	Wild	3	110	634205	USA	Cultivar	3
31	418726[d]	France	Wild	3	111	403847	Canada	Cultivar	3
32	418727[d]	France	Wild	3	112	403851	Canada	Cultivar	3
33	423136[d]	Spain	Wild	1	113	Silver Dollar	USA	Cultivar	3
34	462339[d]	New Zealand	Cultivar	3	116	403886	Canada	Cultivar	3
35	182857	Czech Republic	Uncertain	4	121	418708	Romania	Wild	2
36	189392	New Zealand	Uncertain	3	122	418712	Italy	Wild	3
37	198070	Sweden	Cultivated	3	124	418722	Luxembourg	Wild	3
38	204085	Cyprus	Uncertain	4	127	418741	France	Wild	3
43	225825	Denmark	Uncertain	3	128	420124	Japan	Cultivar	1
46	231566	Libya	Uncertain	4	132	Bright Star SLT	USA	Cultivar	3
49	231576	Algeria	Uncertain	3	133	440474	Former Soviet Union	Wild	2
50	231580	Algeria	Uncertain	3	134	462335	New Zealand	Cultivated	3
136	462337	New Zealand	Cultivated	3	166	598515	Turkey	Wild	2
139	505840	Former Soviet Union	Cultivated	3	168	598518	Turkey	Wild	3
142	505843	Former Soviet Union	Cultivated	3	173	598911	Tunisia	Wild	1
148	577260	UK	Wild	3	175	Divine	USA	Cultivar	3
149	577265	UK	Wild	3	176	Catalina	USA	Cultivar	3
151	577269	Norway	Wild	3	179	610802	Norway	Wild	3
153	Manhattan4	USA	Cultivar	2	180	610802	UK	Wild	3
154	578760	USA	Cultivar	3	182	610925	Tunisia	Wild	1
158	595046	UK	Wild	3	185	610950	Tunisia	Wild	1
159	598434	Italy	Wild	3	187	611036	Russian Federation	Wild	2
161	598441	Switzerland	Wild	3	190	619474	Romania	Cultivated	4
164	598452	UK	Wild	3					

[a] ID number representing accessions used in this study.
[b] Improvement status obtained from USDA germplasm bank.
[c] *Q*, identified population structure groups in this study.
[d] Core collection.

109 published genome-wide SSR markers [27–30] representing seven chromosomes in perennial ryegrass were screened in all accessions. Forward primer sequence was modified by adding an M13 tail (5′-ACG ACG TTG TAA AAC GAC) to the 5′ end, and the M13 primers were labeled with four fluorescent dyes of different colors [FAM (blue), VIC (green), PET (yellow), NED (red)] to facilitate the universal labeling of the PCR products. Each 10 μL PCR reaction consisted of 1×PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.05 μM forward tailed primer, 0.1 μM reverse primer, 0.05 μM fluorescent-labeled M13 primer, 1.0 U *Taq* DNA Polymerase, and 60 ng DNA. All PCR were performed in a 384-well iCycler thermocycler (Bio-Rad Inc., Hercules, CA, USA) using a touch-down program [31]. The amplified fragments were separated in an ABI 3730 DNA Sequencer (Applied Biosystem, Inc., Foster City, CA, USA). Alleles were called using GeneMarker 1.6 software (SoftGenetics, LLC, State College, PA, USA) and checked twice manually for accuracy. The allele sizes differing in at least two base-pairs were considered to be polymorphic between accessions. All confirmed polymorphic alleles were used for population structure and relative kinship analysis.

2.5. Population structure, relative kinship, and LD decay

Population structure (*Q*) was determined by using STRUCTURE 2.3.2 software [32]. The structure was run ten times by setting predefined *k* (the number of group in a population) from 1 to 10 using admixture models, with 10,000 burn-in time and 10,000 iterations of Markov chain convergence for each run. After $k = 4$ was determined as optimal number of groups, ten independent runs were performed by setting *k* from 3 to 6, with a burn-in time of 50,000 and a replication number of 100,000 for each run. This produced the same results as those of the preliminary runs. Among the ten runs at $k = 4$, the one with the highest likelihood value was selected to be

Table 2
Mean squares from analysis of variance for leaf color (Color), chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR) of 99 perennial ryegrass accessions under 7 d of control and submergence treatment.

Sources	Experiment 1				Experiment 2			
	Color	HT	RGR	F_v/F_m	Color	HT	RGR	F_v/F_m
Submergence (*S*)	885.7**	143.4**	37.5*	32.2**	460.5**	123.7**	10.6*	545.9**
Accession (*A*)	10.9**	10.9**	7.3**	3.0**	7.5**	10.4**	9.0**	2.7**
$S \times A$	4.0**	1.8**	1.3	1.7**	3.1**	2.1**	1.5**	1.7**

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

assigned the proportion of membership for each accession. Relative kinship (*K*) among samples was calculated using 109 SSR markers by SPAGeDi software [33], and the Loiselle coefficient [34] was used to create the pair-wise kinship matrix (99×99). Eighty-four SSR markers with known map locations were used for LD decay analysis using PowerMarker 3.25 software [35]. The LD pattern was determined by coefficient of determination (R^2) of SSR, a measurement of correlation between a pair of variables [36].

2.6. Model testing and association mapping

The simple linear, *Q*, *K*, and *Q*+*K* models were tested to identify the best model fitting each individual trait for association mapping in the perennial ryegrass populations using previously recommended procedures [37]. The *Q*+*K* model was tested with a subpopulation membership percentage as fixed covariates and kinship as a random effect [37]. The best model fit for each trait was determined according to the Bayesian Information Criterion (BIC), with the lowest value as the best approximate [37]. The marker–trait association analysis was conducted using TASSEL 2.1 software along with the GLM procedure [38]. The significant threshold for the association was set at $P < 0.01$. The trait data from Exp 1 and Exp 2 were combined for model testing and association mapping.

2.7. Experiment design and statistical analyses

Both experiments were designed in a split plot with two treatments (control and submergence). Each experiment had three replicates arranged in three different benches in a greenhouse. In each bench (replicate), 99 accessions (pots) were randomly assigned into 5 containers within the control or the submerged regime, respectively. Statistical analysis was performed using SAS Proc Mix [39] with both accessions and submergence as a fixed effect. This allowed us to examine the trait differences caused by both submergence and accession effects. The treatment effects and accession differences for each trait were tested using the Least Significant Difference (LSD) test at $P < 0.05$. Error variances among experiments were not homogenous for most parameters, and data from plant response to submergence were presented for each experiment. Similar correlations among traits were found in the two experiments, and therefore, only Pearson correlation coefficients from Exp 2 were presented.

3. Results

3.1. Treatment effects and trait variation

Significant treatment and accession effects and were observed for leaf color, F_v/F_m, HT, and RGR in both experiments (Table 2). Across all accessions, the mean leaf color dropped from 6.0 to 4.0 in Exp 1 and from 6.1 to 2.9 in Exp 2, respectively, under submerged conditions (Table 3). Submergence significantly reduced HT and RGR by 16% and 35%, respectively, in Exp 1, and 24% and 26%, respectively, in Exp 2 compared to their relative controls (Table 3). F_v/F_m was also significantly reduced by submergence, although the reduction was not as large as that of HT and RGR. Large variations in color, F_v/F_m, HT, and RGR values were observed in response to the flooding treatments among the accessions in both experiments (Table 3).

Significant correlations between HT and RGR, F_v/F_m and HT, and F_v/F_m and RGR were found under both the control and submerged conditions (Table 4). The leaf color was significantly correlated with RGR ($r = 0.50$, $P < 0.001$) under the submergence stress, but not under normal growth conditions. High correlations were identified for all the traits between the control and submergence stress, with

Table 3
Range of leaf color, chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR) for 99 perennial ryegrass accessions under 7 d of control (*C*) and submergence (*S*) treatment.

Traits	Experiment 1				Experiment 2			
	Minimum	Maximum	Mean	Std.[a]	Minimum	Maximum	Mean	Std.
C-Color	2.6	9.0	6.0	1.4	3.3	9.0	6.1	1.4
S-Color	1.7	6.0	4.0*	0.85	1.0	7.7	2.9*	1.2
D%[b]	−27.3	63	30.5	14.7	−8.3	84.2	49.3	17.9
C-F_v/F_m	0.76	0.82	0.80	0.01	0.74	0.83	0.82	0.01
S-F_v/F_m	0.74	0.81	0.78*	0.01	0.58	0.78	0.74*	0.04
D%	−3.1	7.3	1.5	1.86	4.2	27.3	9.5	4.4
C-HT (cm)	1.6	14.2	9.0	2.47	2.6	12.8	7.6	2.2
S-HT	2.5	14.1	7.5*	2.04	1.0	10.2	5.8*	1.5
D%	−83	49.7	14.1	20.0	−143.7	64.1	20.3	24.7
C-RGR (mg)	2.6	40.5	19.9	7.99	0.6	33.0	11.7	7.4
S-RGR	3.7	32.6	13.0*	5.53	1.3	23.7	8.6*	5.2
D%	−44.8	70.2	30.8	23.5	−257.6	80.9	9.8	57.5

[a] Standard deviation.
[b] *D*, percentage of reduction = $[(C - S)/C \times 100]$.
* Significant at $P < 0.05$ between *C* and *S* treatment for a given trait.

Table 4
Pearson correlation coefficients among leaf color (Color), chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR) under control (*C*) and submergence (*S*) stress.

	C-Color	*C*-F_v/F_m	*C*-HT	*C*-RGR	*S*-Color	*S*-F_v/F_m	*S*-HT	*S*-RGR
C-Color	1							
C-F_v/F_m	0.23*	1						
C-HT	−0.29**	0.30**	1					
C-RGR	−0.01	0.37***	0.62***	1				
S-Color	0.40***	0.32**	0.04	0.42***	1			
S-F_v/F_m	0.08	0.45***	0.20	0.35***	0.44***	1		
S-HT	−0.27**	0.19	0.70***	0.48***	0.16	0.28**	1	
S-RGR	−0.07	0.36***	0.42***	0.69***	0.50***	0.48***	0.60***	1

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.

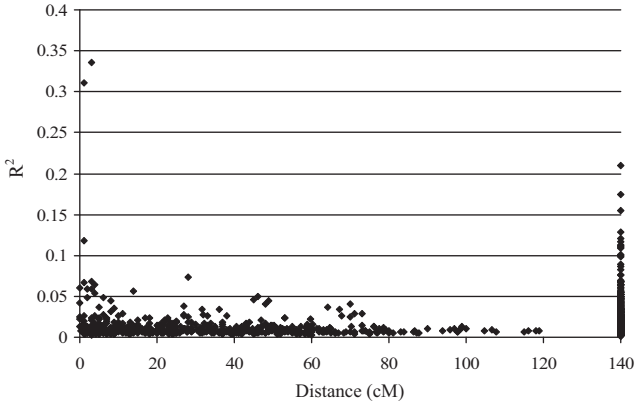

Fig. 1. Pair-wise linkage disequilibrium (LD) (R^2) of 84 SSR markers as a function of the inter-marker distance in cM (with known position on a linkage map of perennial ryegrass). Data points at 140 cM represent LD values between pairs of unlinked markers, while the remaining points represent LD values between pairs of linked markers.

the highest correlations for HT ($r = 0.70$, $P < 0.001$) and RGR ($r = 0.69$, $P < 0.001$).

3.2. LD, population structure, relative kinship, and model testing

A significant LD between 10 pairs of linked SSR markers was found within 4 cM ($R^2 > 0.05$, $P < 0.005$) (Fig. 1). At $R^2 > 0.05$, two pairs of markers linked with genetic distance less than 14 cM and 28 cM, respectively. A total of 45 pairs of unlinked markers were found with significant LD at $R^2 > 0.05$ ($P < 0.005$) (Fig. 1, points at 140 cM).

Four structure groups (G1, G2, G3, and G4) were identified in the collection of perennial ryegrass (Fig. 2). G1 contained 11 accessions, mainly wild and materials with uncertain pedigrees from North Africa (Algeria, Morocco, and Tunisia) and Spain. G2 contained 12 accessions, mainly wild materials from Eastern Europe and Asia. G3 was the largest group, with 65 accessions of mixed origins including materials from Oceania, the U.S., Canada, Europe, and South America, which included the majority of cultivars and cultivated materials. Further distinct subgroups were not detected within the G3 (data not shown). The G4 had 11 accessions, mainly uncertain materials from Europe.

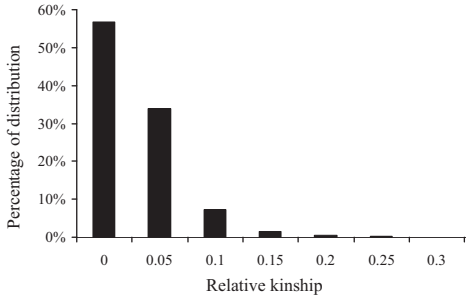

Fig. 3. The distributions of pair-wise kinship coefficients for 99 diverse perennial ryegrass accessions.

Obvious kinship (*K*) was not detected in this population (Fig. 3). An approximate 90% of the pair-wise kinship estimates were between 0 and 0.05. Less than 10% of the pair-wise kinship estimates were from around 0.1 to 0.15, representing some familial relationships.

Among the four models tested, the *Q* model resulted in the smallest Bayesian Information Criterion (BIC) values and thus was selected as the best model for association analysis in this study (Table 5). The simple linear and *K* models had the biggest BIC values, and the mixed *Q* + *K* model was in between.

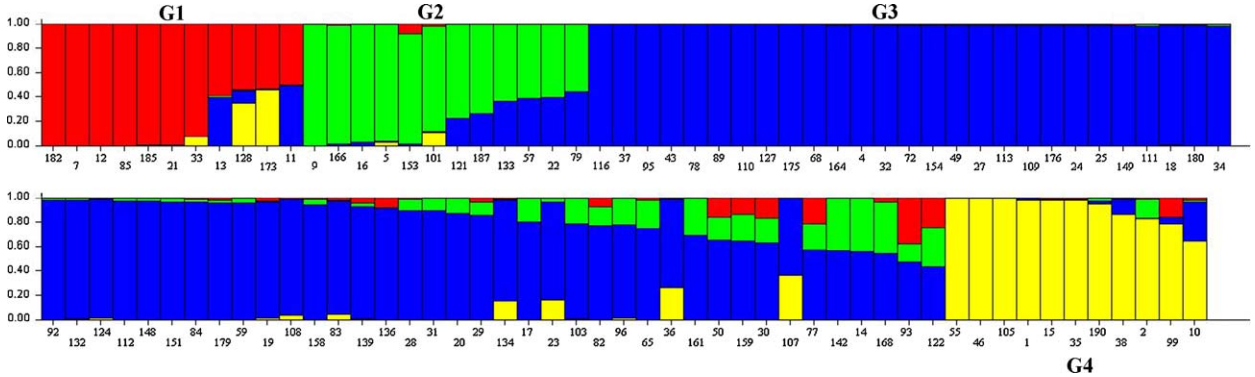

Fig. 2. Population structure analysis of 99 perennial ryegrass accessions. Numbers on the *x*-axis indicate the accession and numbers on the *y*-axis show the group membership. G1, G3, G3, and G4 represent the identified structure groups.

Table 5
Goodness of fit of four different models in explaining phenotypic variation of leaf color (Color), chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR).

Model	*D*-Color[a]		*D*-F_v/F_m		*D*-HT		*D*-RGR	
	−2 Log likelihood	BIC[b]	−2 Log likelihood	BIC	−2 Log likelihood	BIC	−2 Log likelihood	BIC
Simple linear	782.7	787.3	464.0	468.6	822.2	826.8	956.5	961.1
Structure (*Q*)	750.1	754.7	453.3	457.9	802.6	807.2	932.9	937.5
Kinship (*K*)	780.3	789.5	464.0	473.2	822.2	831.4	956.4	965.6
Q+*K*	750.1	759.3	453.2	462.4	802.6	811.7	932.9	942.1

[a] *D*, percentage of reduction between control (*C*) and submergence (*S*): $[(C-S)/C \times 100]$.
[b] Bayesian Information Criterion (smaller is better).

3.3. Association mapping

A total of 23 associations were identified between the SSR markers and the four calculated traits (percentage of reduction) using a simple linear model, while only 15 associations were found using the *Q* model (Table 6). The simple linear and *Q* models did not show any differences in testing association of SSR with HT. The marker B2F1, located in chromosome 5, was associated with leaf color reduction. Three markers, LpACT13H2, LpACT14C9, and B3C11, located in chromosomes 6 or 7, were associated with F_v/F_m reduction. Markers rv0992 and B3C10, located in chromosomes 3 and 7, were associated with RGR reduction. Seven markers, LpSSR085, PRE, LpHCA17C11, LpSSR021, rye014, LpACT43C6, and B4C4 located in chromosomes 1, 2, 6, and 7, were associated with HT. The two markers of LpSSR085 and PRE were both associated with the HT and located in chromosome 1 with an interval of 5 cM (Table 6). Similar results were also observed for markers of LpHCA17C11 and LpSSR021 in chromosome 2 (Table 6).

4. Discussion

Plants survive submergence stress through avoidance or tolerance mechanisms in general [9]. Some flood tolerant perennial grass species exhibit a stimulation of leaf elongation under submergence to restore contact between leaves and air [12,13]. This allows plants to avoid further submergence stress injury. Some plant species use tolerance mechanisms and conserve energy by limiting underwater growth and adjusting metabolism [40]. Plants may balance both strategies to survive, and a flooding regime may be a determinant in selecting one or the other. In this study, submergence reduced leaf green color of perennial ryegrass. However, grasses with relatively darker green color maintained leaf dry weight (RGR), but not HT under the submergence conditions (Table 4). The results suggest that capability of plants to adjust metabolisms is important to the survival of some perennial ryegrass under submergence stress. The dynamics of the level of plant hormone may play an important role in determining plant growth responses to flooding. Bakhtenko et al. [41] found that growth inhibition in wheat and oat (*Avena sativa* L.) during flooding was due to the accumulation of abscisic acid and ethylene, while the repair processes showed the increased level of auxins, cytokinins, and gibberellins. Responses of hormones to submergence stress and how these responses control diverse growth of perennial ryegrass populations under stress deserve further investigation. The wide range of HT and RGR values observed in this study provide a good basis for phenotyping plant growth under normal and stressed conditions. F_v/F_m is an indicator of photochemical efficiency that has been used for determining waterlogging and flooding tolerance [42,43]. Compared to HT and RGR, F_v/F_m exhibited a relatively small range in the population, but significant differences were observed between the two treatments. F_v/F_m was well correlated with color, HT, and RGR under both the control and submerged conditions, suggesting that

Table 6
Association of SSR markers with percentage of reduction of leaf color (Color), chlorophyll fluorescence (F_v/F_m), maximum plant height (HT), and relative growth rate (RGR) of perennial ryegrass accessions.

Trait	Locus	Chromosome no.	Position (cM)	Simple linear model	Structure *Q* model
Color	PRG	4	119	**[a]	
Color	PR10	NA[b]	NA	**	
Color	B2F1	5	28		**
F_v/F_m	B2G6a	1	NA	**	
F_v/F_m	B3D4	1	NA	**	
F_v/F_m	LPSSRH02C11	3	NA	**	
F_v/F_m	PR14	4	116	**	
F_v/F_m	PRG	4	119	**	
F_v/F_m	LpACT13H2	6	26	**	**
F_v/F_m	LpACT14C9	6	NA	**	**
F_v/F_m	B3C11	7	57	**	**
RGR	PR39	1	54	**	**
RGR	B1A2	3	68	**	
RGR	rv0992	3	NA	**	**
RGR	LpACT13H2	6	26	**	
RGR	B3C10	7	80	**	**
RGR	LPSSRH01A07	NA	NA	**	**
HT	LpSSR085	1	47	**	**
HT	PRE	1	52	**	**
HT	LpHCA17C11	2	49.3	**	**
HT	LpSSR021	2	43	**	**
HT	rye014	6	NA	**	**
HT	LpACT43C6	7	NA	**	**
HT	B4C4	7	94	**	**

[a] Significant association at $P < 0.01$.
[b] Not known.

F_v/F_m is a useful parameter for large, fast, and accurate assessment of plant physiological changes under submergence stress.

The resolution of association mapping depends on the structure of LD across the genome [44]. The genome-wide extent of LD in plants could vary across genomes and between species, and LD decay has been reported to occur within 3–50 cM in different crop species [45–47]. Out-breeding species such as perennial ryegrass are expected to show a more rapid LD decay than in-breeding species [48]. Our results on LD decay within 4 cM were consistent with those of the previous study in perennial ryegrass (4.37 cM) using AFLP markers [49]. A rapid LD decay may indicate that a much greater density of markers is needed to identify the association between phenotype and genotype. Thus, the candidate gene-association mapping approach can be a good consideration. Genes controlling flowering time of perennial ryegrass have been successfully identified using the candidate gene-association approach [19]. The results provide evidence of the potential for association mapping of important agronomic traits in perennial ryegrass.

Given the diverse geographical origins, the germplasm panel may contain either population structure (associated with local adaptation or diversifying selection), or familial relatedness (from the recent co-ancestry), or both [15]. Population structure is universal among organisms [50]. One of the reported constraints of association mapping studies is the easy detection of false positives resulting from the existence of the genetic structure in the populations studied [51]. False positive associations of markers with traits could occur if a model is selected without considering the impact of population structures or familial relatedness on the traits within the population. In the structured maize population, the mixed model ($Q+K$) showed a significant improvement in goodness of fit for traits such as flowering time, ear weight, and ear diameter, compared with that of the simple linear, Q, or K models [37]. The perennial ryegrass populations used in this study contained population structure but no obvious familial relationships (Fig. 2). Less than 35% of associations of markers with traits were identified using Q model, compared to the simple linear model (Table 6). No improvement in the model fitness when comparing K or $Q+K$ to Q model demonstrated that the Q model was sufficient to explain the phenotypic variation in submergence responses and eliminate the false association between SSR markers and traits of perennial ryegrass (Fig. 2, Tables 5 and 6). Wang et al. [22] also reported that implementation of Q, K, or both factors reduced significantly the number of markers associated with iron deficiency chlorosis in soybean by 50%, relative to single factor analysis of variance. All the results indicate that model testing for quantitative traits is necessary for increasing accuracy of association.

Little is known about the association of SSR loci with submergence tolerance-related traits in plant species. Fifteen SSRs linked to growth and physiological responses to submergence stress were detected in perennial ryegrass. It is interesting to find that two pairs of markers, LpSSR085 and PRE and LpHCA17C11 and LpSSR021, were linked to the same trait. Thus it is most likely that a QTL for HT located nearby LpSSR085 and PRE in chromosome 1 and nearby LpHCA17C11 and LpSSR021 in chromosome 2. In addition, B3C10 and B4C4, both located in chromosome 7 with 14 cM apart, were associated with different traits of RGR and HT, respectively. Since RGR was correlated with HT, these two markers may link to the same QTL for plant growth in chromosome 7. The marker, LpACT43C6, which was reported to be associated with heading date [30], was correlated with HT in this study, suggesting an interrelationship between heading date and HT as assessed by the SSR. The results indicate that at least three candidate QTL located in these chromosome positions play an important role in upward growth response of perennial ryegrass under submergence stress.

In summary, association mapping has become a powerful tool for identifying genes and markers linked to phenotypic traits. Large variations in F_v/F_m, HT and RGR were found among accessions of perennial ryegrass under submerged conditions. Four subpopulations were identified in the collection but no obvious relative kinship (K) was found. The Q model was the best model to describe associations between SSR and traits. Fifteen SSR markers were associated with a reduction in leaf color, F_v/F_m, HT, or RGR under submergence stress using the Q model. These markers can be used for genetic improvement of submergence tolerance of perennial ryegrass after further validation. To increase mapping resolution in perennial ryegrass, further research is needed to select candidate genes underlying traits and identify single-nucleotide polymorphism (SNPs) for genotyping the mapping population. The diverse stress responses of the perennial ryegrass populations provide a foundation for further association mapping research.

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